



Plugging in or going wireless: strategies for interspecies electron transfer

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Interspecies exchange of electrons enables a diversity of microbial communities to gain energy from reactions that no one microbe can catalyze. The first recognized strategies for interspecies electron transfer were those that relied on chemical intermediates that are recycled through oxidized and reduced forms. Well-studied examples are interspecies H₂ transfer and the cycling of sulfur intermediates in anaerobic photosynthetic communities. Direct interspecies electron transfer (DIET) in which two species establish electrical contact is an alternative. Electrical contacts documented to date include electrically conductive pili, as well as conductive iron minerals and conductive carbon moieties such as activated carbon and biochar. Interspecies electron transfer is central to the functioning of methane-producing microbial communities. The importance of interspecies H₂ transfer in many methanogenic communities is clear, but under some circumstances DIET predominates. It is expected that further mechanistic studies and broadening investigations to a wider range of environments will help elucidate the factors that favor specific forms of interspecies electron exchange under different environmental conditions.

Keywords: syntrophy, diet, interspecies electron transfer, conductive pili, coculture

INTRODUCTION

Interspecies electron transfer plays a key role in the functioning of methane-producing microbial communities, which have a significant impact on the global carbon cycle (Stams and Plugge, 2009; Sieber et al., 2012). Organic matter mineralization to methane by microbial processes contributes to 69% of the atmospheric CH₄ (Conrad, 2009) and it involves four major steps (Figure 1A):

(1) Hydrolytic bacteria break down complex compounds such as polysaccharides, proteins, nucleic acids, and lipids to monomeric substances (Schink and Stams, 2013), (2) Primary fermenters convert monomeric substances to H₂/formate, CO₂ and small organic molecules such as lactate, succinate, fatty acids, and acetate (Morris et al., 2013; Schink and Stams, 2013), (3) Syntrophic bacteria carry out secondary fermentation of small organic molecules to produce acetate, formate, H₂ and CO₂ (Morris et al., 2013; Schink and Stams, 2013), or releases electrons for direct electric connections (Summers et al., 2010; Rotaru et al., 2014), (4) Methanogenic Archaea uses electrons from H₂/formate/shuttles or directly to reduce CO₂ to CH₄ (Morris et al., 2013; Rotaru et al., 2014; Sieber et al., 2014).

Interspecies electron transfer via H₂/formate has been extensively reviewed in recent years (Morris et al., 2013; Schink and Stams, 2013; Sieber et al., 2014). Besides, H₂/formate, there are many important mechanisms of interspecies electron transfer reported, which include but are not limited to pili mediated direct interspecies electron transfer (DIET; Summers et al., 2010; Morita et al., 2011; Nagarajan et al., 2013; Shrestha et al., 2013a,b; Rotaru et al., 2014) and mineral mediated direct interspecies

electron transfer (Kato et al., 2012a,b; Liu et al., 2012, 2014; Chen et al., 2014), or by shuttle molecules like cysteine (Kaden et al., 2002), sulfur compounds (Biebl and Pfennig, 1978; Milucka et al., 2012), and humics (Lovley et al., 1999; Liu et al., 2012). This review discusses recent findings on interspecies electron transfer during syntrophic interactions, with the main focus on DIET mechanisms.

H₂ AND FORMATE AS ELECTRON TRANSFER MOLECULES

H₂ and formate are important electron transfer molecules that are reported in various methanogenic environments (Schink and Stams, 2006, 2013; Stams and Plugge, 2009), these are described briefly under separate headings below:

H₂ AS ELECTRON TRANSFER MOLECULE

Interspecies electron transfer via H₂ was first demonstrated almost four decades ago in a defined co-culture (Bryant et al., 1967) of the “S organism,” which converted ethanol to acetate and H₂, only in the presence of *Methanobacterium ruminantium*, which consumed H₂ for the reduction of CO₂ to CH₄ (Bryant et al., 1967). H₂ is a very powerful electron donor under anoxic conditions and must be continuously removed by partner organism in order for the syntrophic interaction to take place (Nedwell and Banat, 1981; Lovley and Ferry, 1985; Kleerebezem et al., 1999; Wintermute and Silver, 2010). The generation of H₂ is energetically unfavorable at H₂ partial pressures above 10⁻³ bar (Schink and Stams, 2013), however, syntrophic microorganisms bypass this energetic barrier by coupling the unfavorable H₂ production with

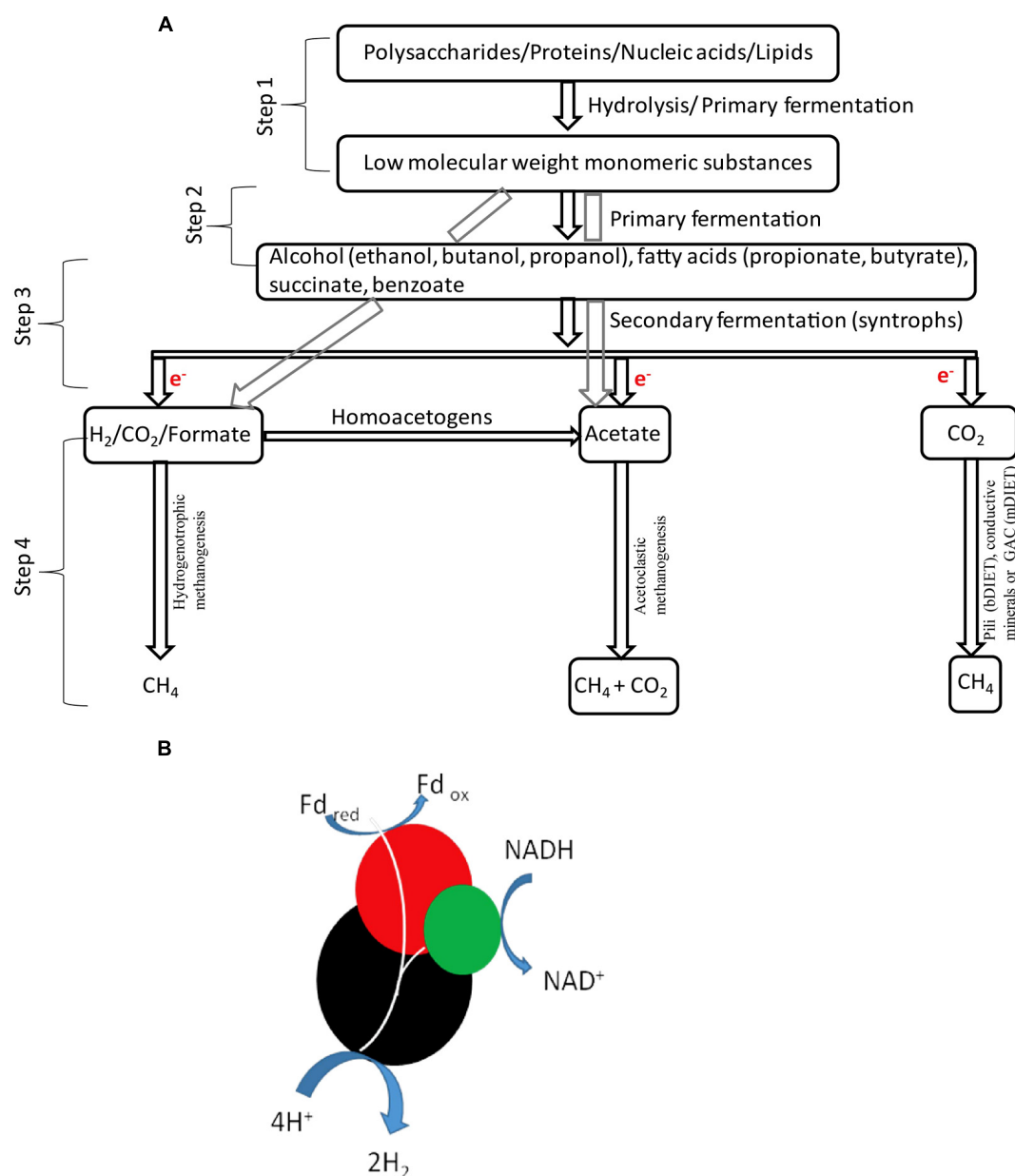


FIGURE 1 | Organic matter degradation in methanogenic environments (A). Sketch of the coupling of H₂ with the energetically favorable oxidation of a reduced ferredoxin in the presence of putative NADH-linked confurcating hydrogenases [modified from McNerney et al., 2011; **(B)**].

the energetically favorable oxidation of a reduced compound like ferredoxin (**Figure 1B**), a process known as electron confurcation (Schut and Adams, 2009; Sieber et al., 2010, 2012). Confurcating hydrogenases are found in the genomes of all H₂ generating syntrophs described to date (Sieber et al., 2010, 2012).

FORMATE AS ELECTRON TRANSFER MOLECULE

Formate is an alternative to H₂ and could also act as an electron carrier between syntrophic partners (Thiele and Zeikus, 1988; Boone et al., 1989; Hattori et al., 2001; de Bok et al., 2004;

Stams et al., 2006; Stams and Plugge, 2009). The use of formate as an electron transfer molecule has been noticed especially in co-cultures thriving on proteins (Zindel et al., 1988) or fatty acids like propionate and butyrate (de Bok et al., 2004; Sousa et al., 2007). Certain communities might favor formate transfer because formate has ca. three times higher diffusion coefficient as compared to H₂, and allows larger mass transfer to methanogens (Boone et al., 1989). It has been also reported that some syntrophic interactions uses both formate and H₂ to transfer electrons between species (Boone et al., 1989; Dong and Stams, 1995; Stams et al., 2006; Rotaru et al., 2012). This dual mechanism of electron

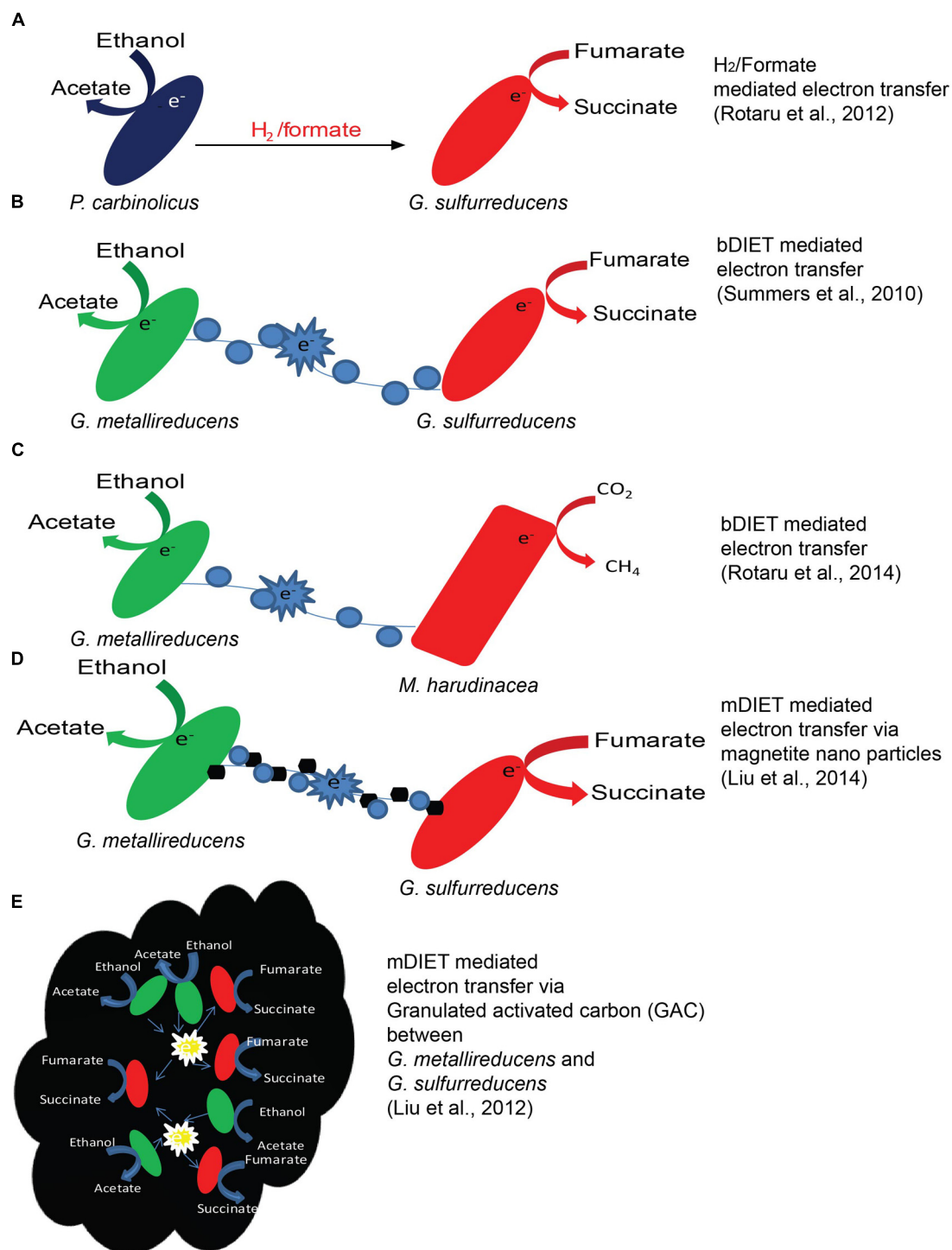


FIGURE 2 | Examples of mechanisms of electron transfer. H_2 transfer between *P. carbinolicus* and *G. sulfurreducens* (A), bDIET between *G. metallireducens* and *G. sulfurreducens* (B), mineral mediated mDIET between *G. metallireducens* and *G. sulfurreducens* with nano-sized minerals (D) or GAC (E) in the presence of ethanol as the electron

donor and fumarate as the electron acceptor. DIET in a co-culture of *G. metallireducens* and *Methanosaeta harudinacea* where ethanol was used as electron donor and CO_2 is reduced to CH_4 by *Methanosaeta* using electrons received directly from *G. metallireducens* via bDIET (C).

transfer using H_2 and formate (Figure 2A) has been studied in detail using deletion mutants, in a co-culture of *Pelobacter carbinolicus* and *Geobacter sulfurreducens* (Rotaru et al., 2012). For example, when a co-culture was established with a hydrogenase mutant (*hybL*) of *G. sulfurreducens*, the formate dehydrogenase (*fdnG*) gene of *G. sulfurreducens* was over-expressed (Rotaru et al., 2012).

ELECTRON TRANSFER VIA SHUTTLE MOLECULES

Electron shuttles are chemical compounds that facilitates the transfer of electrons to and from bacteria these may include sulfur compounds (Biebl and Pfennig, 1978), humic substances (Lovley et al., 1996, 1998, 1999; Newman and Kolter, 2000), and flavins (Marsili et al., 2008; von Canstein et al., 2008; Brutinel and Gralnick, 2012), etc.

SULFUR COMPOUNDS AS MEDIATORS FOR INTERSPECIES ELECTRON TRANSFER

Sulfur compounds as shuttle were first discovered between green sulfur bacteria and sulfate-reducing bacteria (SRB; Biebl and Pfennig, 1978). $S(0)$ is converted to sulfide by a sulfate reducing bacteria and then recycled back to $S(0)$ by a photosynthetic green-sulfur bacteria creating an interspecies S-cycle (Biebl and Pfennig, 1978). The second discovered S-based interspecies interaction used cysteine as electron shuttle between *G. sulfurreducens* and *Wolinella succinogenes*, growing with acetate as electron donor and nitrate as electron acceptor (Kaden et al., 2002). S-compounds were also found responsible for electron transfer between anaerobic methane oxidizing *Archaea* (ANME) and sulfate reducing bacteria (Boetius et al., 2000), which oxidizes methane with sulfate, one of the most studied, yet least understood interactions. The members of the anaerobic oxidation of methane consortia were initially thought to exchange electrons via methyl-sulfides (Moran et al., 2008), however, more recently the electron carrier within the consortium was revealed to be polysulfides (Milucka et al., 2012).

HUMICS AND HUMICS EQUIVALENTS AS ELECTRON SHUTTLES

Humic substances are ubiquitous in nature (Lovley et al., 1996; Bittner et al., 2007). The humic substance analog, anthraquinone disulphonate (AQDS) serves as an electron shuttles between *G. metallireducens* and *G. sulfurreducens* (Liu et al., 2012), or between *G. metallireducens* and *W. succinogenes* (Lovley et al., 1999). This came as no surprise because it is known that certain microorganisms can use AH_2QDS as electron donor (Lovley et al., 1999), while others use AQDS as electron acceptor (Lovley et al., 1996). However, AQDS cannot mediate electron transfer in *G. metallireducens* and *M. barkeri* co-cultures, likely because of the redox potential of the AQDS couple is too high to reduce carbon ($E_0' = -184$ mV) to reduce carbon dioxide to methane ($E_0' = -240$ mV; Liu et al., 2012).

FLAVINS AS ELECTRON SHUTTLES

Flavins were also noted to improve electron transfer to electrodes in *Shewanella* biofilms (Marsili et al., 2008; von Canstein et al., 2008; Brutinel and Gralnick, 2012) yet their impact on interspecies interactions remains to be reported.

DIRECT INTERSPECIES ELECTRON TRANSFER

To clearly distinguish between conductive mineral mediated DIET and direct cell contact DIET, we have subcategorized the pili mediated electron transfer, as biological DIET (bDIET), and the conductive mineral mediated DIET, as mineral DIET (mDIET).

BIOLOGICAL DIET

Biological DIET (Figures 2B,C) was first described in *G. metallireducens* and *G. sulfurreducens* co-cultures, growing in a defined minimal medium with ethanol as electron donor and fumarate as electron acceptor (Summers et al., 2010). Tightly associated aggregates were consistently noticed in co-cultures growing via bDIET (Summers et al., 2010; Shrestha et al., 2013a; Rotaru et al., 2014) but not during growth via H_2 /formate electron transfer (Rotaru et al., 2012). The mechanism for bDIET in *Geobacter* co-cultures was intensely studied during the past few years, combining phenotypic, genetic, transcriptomics, proteomics analysis (Summers et al., 2010; Shrestha et al., 2013a,b). bDIET might be favored over H_2 or formate transfer under certain conditions (Lovley, 2011) as demonstrated using genome-scale models including genomic, transcriptomic and physiological data (Nagarajan et al., 2013). The absence of H_2 /formate mediated electron transfer in the co-culture was best shown by the ability of *G. metallireducens* to generate successful syntrophic co-cultures with a double mutant of *G. sulfurreducens* ($\Delta hybL \Delta fdnG$) incapable of H_2 or formate uptake (Rotaru et al., 2012). Furthermore, bDIET is seemingly capable to produce successful co-cultures in the absence of acetate transfer as supportive mechanism of electron exchange as revealed in a recent study (Shrestha et al., 2013a) in co-cultures of *G. metallireducens* with strain of *G. sulfurreducens* depleted in acetate utilization capacity, a citrate synthase mutant ($\Delta gltA$; Ueki and Lovley, 2010). This study clearly revealed that bDIET alone is sufficient for energy conservation in syntrophic co-cultures.

Biological DIET interactions with fumarate as terminal electron acceptors are probably not ecologically relevant, but more recently bDIET was discovered in co-cultures of *G. metallireducens* with *Methanosaeta harudinacea* (Rotaru et al., 2014). These two genera of methanogens are responsible for the majority of methane emission in environments such as paddy soils (Grosskopf et al., 1998; Feng et al., 2013) or anaerobic digesters (Vavilin et al., 2008; Morita et al., 2011; Rotaru et al., 2014; Ying et al., 2014). Only these acetoclastic methanogens were capable of bDIET-interactions with *G. metallireducens*, whereas hydrogenotrophic methanogens were not (Rotaru et al., 2014). *Methanosaeta* was shown to use electrons directly for the reduction of CO_2 to methane because the methanogen converted 1/3 of the ^{14}C -bicarbonate to ^{14}C methane (Rotaru et al., 2014). Other shuttles were excluded as electron transferring mechanisms because a pili-deficient *G. metallireducens* could not produce successful co-cultures with *Methanosaeta* or *Methanosarcina* (Rotaru et al., 2014).

Role of pili in bDIET

Pili are known to have an important role in biofilm formation (Moreira et al., 2006; Reguera et al., 2007; Oxaran et al., 2012; Snider et al., 2012), but also for the conductive properties of *Geobacter* biofilms (Summers et al., 2010; Malvankar

et al., 2011; Malvankar and Lovley, 2012, 2014; Vargas et al., 2013). Co-cultures do not grow when initiated with a strain of either *G. metallireducens* (Summers et al., 2010) or *G. sulfurreducens* (Rotaru et al., 2014) in which the gene for PilA is deleted, confirming the importance of conductive pili (Reguera et al., 2005, 2006; Lovley, 2011; Malvankar et al., 2011) networks for bDIET. It has been proposed that the stacking of π - π orbitals of five aromatic amino-acids in the carboxyl-terminus of PilA, the pilin monomer, contribute to the metallic-like conductivity similar to that of conductive organic polymers (Vargas et al., 2013). A *G. sulfurreducens* strain deficient in the five aromatic amino acids (ARO5), the pili were still produced with properly localized OmcS and yet the biofilms of ARO5 showed greatly diminished conductivity (Vargas et al., 2013). In another study, the gene for conductive pili in *G. sulfurreducens* was replaced with the non-conductive *pilA* gene of *Pseudomonas aeruginosa* PAO1 (Liu et al., 2013) generating a mutant strain PAO1, which can express properly assembled *P. aeruginosa* pili ornamented by outer surface c-type cytochromes. However, PAO1 biofilms had significantly lower conductivity than wild type *G. sulfurreducens* and was unable to reduce Fe^{3+} -oxides or produce current (Liu et al., 2013). The lack of conductivity in PAO1 biofilms indicates that three out of five aromatic amino acids at the C-terminus domain are necessary for conductivity (Liu et al., 2013). These findings validated that OmcS alone on scaffold-pili is insufficient to confer conductivity to *Geobacter* biofilms, in contrast to a recent hypothesis, which suggested that conductivity is the result of electron-hopping via cytochromes aligned on the pili of *G. sulfurreducens* (Strycharz-Glaven et al., 2011).

Role of cytochromes in bDIET

Geobacter sulfurreducens was used as model organism for the study of extracellular electron transfer, and several studies revealed that besides pili, *G. sulfurreducens* require a multitude of extracellular and periplasmic cytochromes for insoluble Fe^{3+} oxide reduction (Lloyd et al., 2003; Butler et al., 2004; Qian et al., 2007, 2011; Aklujkar et al., 2009; Lovley et al., 2011; Lovley, 2012), current production (Nevin et al., 2009; Inoue et al., 2010), or current uptake on electrodes (Holmes et al., 2006; Strycharz et al., 2011). However, there are slight differences in the types of cytochromes expressed during growth in electron-donating and electron up-taking modes (Strycharz et al., 2011).

Geobacter sulfurreducens growing via bDIET with *G. metallireducens* highly expresses an extracellular c-type cytochrome, OmcS (Summers et al., 2010; Shrestha et al., 2013a,b). OmcS decorates the pili of *G. sulfurreducens* (Leang et al., 2010; Summers et al., 2010) and is required for bDIET and Fe^{3+} reduction (Mehta et al., 2005; Ding et al., 2008; Qian et al., 2011) but not for current production (Nevin et al., 2009). OmcS is not necessary while growing via H_2 interspecies transfer with *P. carbinolicus* (Rotaru et al., 2012).

Another extracellular cytochrome OmcZ, which helps *G. sulfurreducens* achieve high current densities in single species biofilms (Nevin et al., 2009; Richter et al., 2009), was not required for bDIET in *G. sulfurreducens* – *G. metallireducens* co-cultures (Shrestha et al., 2013b) or during iron oxide reduction (Nevin et al., 2009).

There is no correspondence between the well studied extracellular cytochromes in *G. sulfurreducens* and *G. metallireducens*, and today we have yet no clear understanding, about the exact role of each cytochrome in *G. metallireducens* during extracellular electron transfer processes. And yet it must be noted that extracellular cytochrome like OmcS in the electron acceptor strain, *G. sulfurreducens* were highly relevant for the interspecies association. How exactly they aid the electron transfer process is yet to be uncovered.

bDIET in environmental communities

The possible existence of bDIET in the natural ecosystem was first reported by Morita et al. (2011), while studying the mechanism of interspecies electron exchange in the natural methanogenic communities that formed conductive aggregates in a simulated anaerobic wastewater digester converting brewery wastes to methane. The microbial community structure in up-flow anaerobic sludge blanket digester aggregates showed the predominance of *Geobacter* spp. (Morita et al., 2011; Rotaru et al., 2014). It is interesting to note that in most of the methanogenic environments where bDIET is reported, *Geobacter* spp. are abundant (Kato et al., 2012a; Aulenta et al., 2013; Zhou et al., 2013a; Rotaru et al., 2014), which is probably because *Geobacter* spp. form conductive networks using pili (Malvankar et al., 2011; Malvankar and Lovley, 2012) and transfer electrons to methanogens such as *Methanosaeta* (Morita et al., 2011; Rotaru et al., 2014). Similar species abundance has also been reported in enrichment culture converting coal to methane, where *Geobacter* and *Methanosaeta* were the dominant genera (Jones et al., 2010) possibly using coal as an electron donor and an electron transfer mediator.

MINERAL MEDIATED DIET (mDIET)

The need to produce biological conductive molecular networks can be averted by the addition of conductive minerals (Liu et al., 2012, 2014). mDIET could take place via non-biological conductive networks of semi-conductive minerals (Figures 2D,E) like nano-magnetite (Kato et al., 2012a,b; Liu et al., 2014), granulated activated carbon (GAC; Liu et al., 2012) or biochar (Chen et al., 2014) in the absence of molecular conduits.

For example, electrically conductive magnetite nano-particles facilitate mDIET from *G. sulfurreducens* to *Thiobacillus denitrificans*, accomplishing acetate oxidation coupled to nitrate reduction (Kato et al., 2012b). Recently, magnetite nano-particles were shown to compensate for the absence of OmcS on the pili of a deficient *G. sulfurreducens* co-cultured with *G. metallireducens* in the presence of ethanol and fumarate (Liu et al., 2014; Figure 2D). Another conductive material, GAC promotes mDIET, bypassing biologically produced electrical conduits (Liu et al., 2012), as evident from the ability to restore syntrophic metabolism in co-cultures deficient in pili or cytochromes (Liu et al., 2012).

mDIET in environmental communities

Although extracellular appendages are required for the respiration of extracellular electron acceptors (Reguera et al., 2005; Tremblay et al., 2012), they can be replaced with conductive materials which can mediate electron transfer between cells during mDIET. Naturally occurring minerals could offer ecological advantages

because of their abundance in natural ecosystems (Kato et al., 2012b), where they could aid mDIET in the absence of pre-evolved molecular conduits. Iron is one of the most ubiquitous metals in Earth's crust (Braunschweig et al., 2013) and could act as conductive mediator for mDIET, demanding less energetic investment from the species exchanging electrons because there would be no need to produce extracellular components for biological electrical connections (Kato et al., 2012b). For example, magnetite, a conductive iron (II&III)-oxide, stimulated methane production in rice paddy soils and enriched for *Geobacter* and *Methanosarcina* species, which likely exchanged electrons via magnetite minerals (Kato et al., 2012a; Zhou et al., 2013b). Electrically conductive magnetite (Fe₃O₄) nano-particles could also enhance reductive dechlorination of trichloroethane, an ubiquitous groundwater pollutant, by allowing electrons to be transferred extracellular from acetate oxidizing microorganisms to trichloroethane dechlorinating microorganisms (Aulenta et al., 2013). In this study the abundant microorganisms were also *Geobacter* spp., which accounted for 50% of the total bacterial population (Aulenta et al., 2013).

Similarly, it has been reported that poorly crystalline akaganeite (β -polymorph of FeOOH) enhanced mDIET to methanogens in slurries from river sediments (Jiang et al., 2013). In such slurries, *Clostridium* coupled Fe³⁺-akaganeite reduction to Fe²⁺ with acetate oxidation. Partly, electrons from Fe²⁺ were used by the methanogen to convert bicarbonate to methane. Partly, Fe²⁺ ions were re-adsorbed onto akaganeite nano-rods, followed by re-precipitation as structural Fe³⁺ with the simultaneous formation of goethite (α -polymorph of FeOOH) nanofibres (Jiang et al., 2013).

Anthraquinone disulphonate was also suggested to facilitate mDIET between *Geobacter* spp. and *Methanosarcina* spp. in rice paddies (Zhou et al., 2013b). The impact of AQDS on methanogenesis is in contrast with studies in defined co-cultures of *Geobacter* and *Methanosarcina* (Liu et al., 2012). However, soils are not well-defined systems, and it is possible that in soil other interactions happen between humics and soil components, which should be further investigated.

IMPLICATIONS

The electron exchange between syntrophic partners growing together by bDIET requires cells to develop efficient conductive biological contacts via pili and cytochromes in the absence of conductive mediators (mDIET). However, little is known about the importance of bDIET/mDIET-based interactions in the environment or in man-made systems. A better understanding could help devise better strategies for wastewater digestion, or to control methane emission in environments where such emission are high, like landfills, or rice paddies.

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